Unsaturated fatty acids lactose esters: cytotoxicity, permeability enhancement and antimicrobial activity

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Abstract

Sugar based surfactants conjugated with fatty acid chains are an emerging broad group of highly biocompatible and biodegradable compounds with established and potential future applications in the pharmaceutical, cosmetic and food industries. In this work, we investigated absorption enhancing and antimicrobial properties of disaccharide lactose, mono-esterified with unsaturated fatty acids through an enzymatic synthetic approach. After chemical and cytotoxicity characterizations, their permeability enhancing activity was demonstrated using intestinal Caco-2 monolayers through transepithelial electrical resistance (TEER) and permeability studies. The synthesised compounds, namely lactose palmitoleate (URB1076) and lactose nervonate (URB1077), were shown to exhibit antimicrobial activity versus eight pathogenic species belonging to Gram-positive, Gram-negative microorganisms and fungi.

Keywords: sugar-based surfactants, lactose esters, palmitoleic acid, nervonic acid, permeability enhancers, antimicrobial agents.

1. Introduction

Over the past few decades there has been a growing interest on sugar-based surfactants due to the large range of applications, from the biomedical field to cosmetics and food industries [1,2]. This class of molecules are generally classified as biocompatible and biodegradable non-ionic surfactants with emulsifying and antimicrobial abilities [3,4]. Their surface-active properties and applications are mainly influenced by the nature of the sugar headgroup (e.g. mono-, di- or polysaccharides), the carbon chain length and the degree of substitution [5].

The increasing demand for healthy and non-toxic additives has intensified the need for, and research on, novel compounds for food, medical and pharmaceutical applications. In this context, the development of sugar-fatty acid esters is becoming increasingly attractive. Among their possible applications, absorption-enhancing potential for biologics delivery has been recently evaluated [6,7].

Biological therapeutics (biologics) have and will continue to have a major impact on the management of a number of diseases. While their therapeutic potential is often unmatched by small drug molecules, biologics suffer from injection-only administration. Non-invasive delivery of this class of therapeutics is highly attractive. However, drug delivery technologies, which offer the possibility to achieve safe and clinically relevant non-invasive delivery of biologics, are currently lacking. The key challenge to achieving this is a poor permeation of therapeutic macromolecules across the mucosal surfaces [8], which have evolved as biological structures presenting a barrier to the movement of material from the external environment into the systemic circulation.

The use of absorption enhancing agents is a common approach utilised to improve mucosal absorption (and hence the resulting bioavailability) of biologics following mucosal administration [8–11]. While the use of absorption enhancing agents offers significant potential in enabling non invasive delivery of biologics, ‘absorption enhancers’, which are chemically diverse compounds exerting their absorption-enhancing effect through different mechanism(s), have often been associated with unacceptable toxicity profile [12]. Absorption enhancers that are capable of improving the mucosal absorption of biotherapeutics in a safe and therapeutically-effective manner are highly desirable, but the search for these continues [13–15].

In this study we synthetized and characterized lactose palmitoleate and lactose nervonate, two new biodegradable lactose esters based on unsaturated fatty acids, namely palmitoleic (C16:1ω7) and nervonic (C24:1ω9) acids. The cytotoxicity of these compounds was evaluated in vitro and associated to the capacity to act as oral absorption enhancers of biotherapeutics as tested on the intestinal Caco-2 monolayers. Additionally, the compounds were also evaluated for antimicrobial activity by testing minimum inhibitory concentration (MIC) and effect on the growth inhibition of several pathogenic microorganisms.

2. Experimental section

2.1 Chemicals, materials and methods.

Palmitoleic acid and nervonic acid were purchased from TCI, lactose monohydrate from Carlo Erba, while Lipozyme® (immobilized from *Mucor miehei*), *p*-toluenesulfonic acid, 2,2-dimethoxypropane, tetrafluoroboric acid diethyl ether complex and all organic solvents used in this study were purchased from Sigma. Prior to use, acetonitrile was dried with molecular sieves with an effective pore diameter of 4 Å and toluene was saturated with water. Caco-2 cells were obtained from the European Collection of Cell Cultures. Dulbecco's Modified Eagles Medium (DMEM), Hank's Balanced Salt Solution (HBSS, with sodium bicarbonate and without phenol red), non-essential amino acids (100%), L-glutamine (200 mM), fetal bovine serum (FBS), antibiotic/antimycotic solution (10–12,000 U/mL penicillin, 10–12 mg/mL streptomycin, 25–30 μg/mL amphotericin B), trypsin–EDTA solution (2.5 mg/mL trypsin, 0.2 mg/mL EDTA) and fluorescein isothiocyanate-labelled ovalbumin (FITC-OVA) were supplied by Sigma (Poole, UK). MTS reagent, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (commercially known as CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay) was purchased from Promega (USA). Tissue culture flasks (75 cm^3 with ventilated caps), black 96-well plates and Transwell® inserts (12 mm diameter, 0.4 μm pore size, were purchased from Corning (USA). All other chemicals (reagent grade) were purchased from Sigma. Ultrapure chitosan chloride of 213 kDa average molecular weight ('Protasan UP CL 213') was obtained from Novamatrix (Denmark). Thermal analysis was carried out using differential scanning calorimetry (DSC). DSC analysis was performed using a DSC 8500 (Perkin-Elmer, Norwalk, USA) equipped with an

intracooler (Intracooler 2, Perkin-Elmer, Norwalk, USA) and analyzed in an inert N_2 atmosphere. The structures of compounds were unambiguously assessed by MS, ^{1}H NMR, ^{13}C NMR, and IR. ESI-MS spectra were recorded with a Waters Micromass ZQ spectrometer in a negative or positive mode using a nebulizing nitrogen gas at 400 L/min and a temperature of 250 °C, cone flow 40 mL/min, capillary 3.5 Kvolts and cone voltage 60 V; only molecular ions $[M-H]^-$ or $[M+NH_4]^+$ are given. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC 400 or 101, respectively, spectrometer and analyzed using the TopSpin software package. Chemical shifts were measured by using the central peak of the solvent. IR spectra were obtained on a Nicolet Atavar 360 FT spectrometer. Column chromatography purifications were performed under "flash" conditions using Merck 230–400 mesh silica gel. TLC was carried out on Merck silica gel 60 F254 plates, which were visualized by exposure to ultraviolet light and by exposure to an aqueous solution of ceric ammonium molibdate.

2.2 Synthesis of lactose-based surfactants

2.2.1 General procedure for the synthesis of lactose tetra acetate esters (*Z*)-6'-*O*-hexadec-9-enoyl- and (*Z*)-6'-*O*-tetracos-15-enoyl-4-*O*-(3',4'-*O*-isopropylidene-β-D-galactopyranosyl)-2,3:5,6-di-*O*-isopropylidene-1,1-di-*O*-methyl-D-glucopyranose (3a,b).

Lipozyme® (0.078 g) was added to a solution of palmitoleic acid (**1a**) or nervonic acid (**1b**) (0.79 mmol) and 4-*O*-(3',4'-*O*-isopropylidene-β-D-galactopyranosyl)-2,3:5,6-di-*O*-isopropylidene-1,1-di-*O*-methyl-D-glucopyranose (lactose tetra acetate, LTA) [16] (**2**) (0.401 g, 0.79 mmol) in water-satured toluene at 25 °C. The mixture was stirred at 75 °C for 12 h, cooled, diluted with acetone, then filtered, and the filtrate was concentrated. The purification of the residue by column chromatography (petroleum ether/EtOAc 7:3) gave **3a,b** as pale yellow oils.

3a. Yield: 70% (0.413 g). ESI-MS: *m/z* 744 $(M-H)^-$, 763 $(M+NH_4)^+$. ^{1}H NMR (CD_3OD) δ: 0.93 (t, 3H, J = 6.7 Hz, CH_3), 1.30–1.38 (m, 22H), 1.39 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 1.44 (s, 3H, CH_3), 1.49 (s, 3H, CH_3), 1.59–1.70 (m, 2H, CH_2CH_2COOR), 2.03–2.06 (m, 4H, $CH_2CH{=}CHCH_2$), 2.40 (t, 2H, J = 7.0 Hz, CH_2COOR), 3.45–3.47 (m, 6H, 2 -OCH_3), 3.47 (dd, 1H, $J_{8\text{-}9}$ = 7.1 Hz, $J_{8\text{-}7}$ = 8.0 Hz, H^8), 3.91 (dd, 1H, $J_{4\text{-}3}$ = 1.2 Hz, $J_{4\text{-}5}$ = 5.0 Hz, H^4), 4.04 (ddd, 1H, $J_{11\text{-}12a}$ = 1.5 Hz, $J_{11\text{-}}$

$_{10}$ = 2.2 Hz, $J_{11\text{-}12b}$ = 6.8 Hz, H^{11}), 4.05 (dd, 1H, $J_{6b\text{-}5}$ = 6.0 Hz, $J_{6b\text{-}6a}$ = 8.7 Hz, H^{6b}), 4.08 (dd, 1H, $J_{9\text{-}10}$ = 5.5 Hz, $J_{9\text{-}8}$ = 7.1 Hz, H^{9}), 4.14 (dd, 1H, $J_{3\text{-}4}$ = 1.2 Hz, $J_{3\text{-}2}$ = 7.5 Hz, H^{3}), 4.17 (dd, 1H, $J_{6a\text{-}5}$ = 6.0 Hz, $J_{6a\text{-}6b}$ = 8.7 Hz, H^{6a}), 4.22 (dd, 1H, $J_{10\text{-}11}$ = 2.2 Hz, $J_{10\text{-}9}$ = 5.5 Hz, H^{10}), 4.27 (dd, 1H, $J_{12b\text{-}11}$ = 6.8 Hz, $J_{12b\text{-}12a}$ = 11.5 Hz, H^{12b}), 4.30 (dd, 1H, $J_{12a\text{-}11}$ = 1.5 Hz, $J_{12a\text{-}12b}$ = 11.5 Hz, H^{12a}), 4.31 (ddd, $J_{5\text{-}4}$ = 5.0 Hz, $J_{5\text{-}6a} \cong J_{5\text{-}6b}$ = 6.0 Hz, H^{5}), 4.41 (d, 1H, $J_{1\text{-}2}$ = 6.2 Hz, H^{1}), 4.51 (d, 1H, $J_{7\text{-}8}$ = 8.0 Hz, H^{7}), 4.51 (dd, 1H, $J_{2\text{-}1}$ = 6.2 Hz, $J_{2\text{-}3}$ = 7.5 Hz, H^{2}), 5.35 (ddd, 1H, $J_{22\text{-}23a} \cong J_{22\text{-}23b}$ = 6.0 Hz, $J_{22\text{-}21}$ = 11.0 Hz, CH=CH), 5.39 (ddd, 1H, $J_{21\text{-}20a} \cong J_{21\text{-}20b}$ = 6.0 Hz, $J_{21\text{-}22}$ = 11.0 Hz, CH=CH) ppm. ^{13}C NMR (CD_3OD) δ: 13.0, 22.3, 24.2, 24.6, 25.1, 25.5, 25.7, 26.2, 26.7, 26.8, 27.0, 28.6, 28.76, 28.81, 28.9, 29.39, 29.43, 31.5, 33.5, 53.0, 55.1, 63.1, 65.5, 70.8, 73.3, 73.5, 75.4, 76.4, 76.8, 77.5, 79.4, 103.1, 105.7, 108.5, 109.7, 109.8, 129.4, 129.5, 173.8 ppm. IR (Nujol): 2952, 1729, 1712 cm^{-1}.

3b. Yield: 47% (0.222 g). ESI-MS: *m/z* 856 $(M\text{-}H)^-$, 875 $(M+NH_4)^+$. ^{1}H NMR (CD_3OD) δ: 0.93 (t, 3H, *J* = 6.7 Hz, CH_3), 1.30–1.38 (m, 38H), 1.39 (s, 3H, CH_3), 1.41 (s, 3H, CH_3), 1.44 (s, 3H, CH_3), 1.49 (s, 3H, CH_3), 1.59–1.70 (m, 2H, CH_2CH_2COOR), 2.03–2.08 (m, 4H, $CH_2CH{=}CHCH_2$), 2.40 (t, 2H, *J* = 7.0 Hz, CH_2COOR), 3.45–3.47 (m, 6H, 2 -OCH_3), 3.48 (dd, 1H, $J_{8\text{-}9}$ = 7.1 Hz, $J_{8\text{-}7}$ = 8.0 Hz, H^{8}), 3.91 (dd, 1H, $J_{4\text{-}3}$ = 1.2 Hz, $J_{4\text{-}5}$ = 5.0 Hz, H^{4}), 4.04 (ddd, 1H, $J_{11\text{-}12a}$ = 1.5 Hz, $J_{11\text{-}10}$ = 2.2 Hz, $J_{11\text{-}12b}$ = 6.9 Hz, H^{11}), 4.05 (dd, 1H, $J_{6b\text{-}5}$ = 6.0 Hz, $J_{6b\text{-}6a}$ = 8.7 Hz, H^{6b}), 4.08 (dd, 1H, $J_{9\text{-}10}$ = 5.6 Hz, $J_{9\text{-}8}$ = 7.1 Hz, H^{9}), 4.14 (dd, 1H, $J_{3\text{-}4}$ = 1.2 Hz, $J_{3\text{-}2}$ = 7.5 Hz, H^{3}), 4.17 (dd, 1H, $J_{6a\text{-}5}$ = 6.0 Hz, $J_{6a\text{-}6b}$ = 8.7 Hz, H^{6a}), 4.21 (dd, 1H, $J_{10\text{-}11}$ = 2.2 Hz, $J_{10\text{-}9}$ = 5.5 Hz, H^{10}), 4.27 (dd, 1H, $J_{12b\text{-}11}$ = 6.9 Hz, $J_{12b\text{-}12a}$ = 11.5 Hz, H^{12b}), 4.29–4.33 (m, 2H, H^{5}, H^{12a}), 4.41 (d, 1H, $J_{1\text{-}2}$ = 6.2 Hz, H^{1}), 4.51 (d, 1H, $J_{7\text{-}8}$ = 8.0 Hz, H^{7}), 4.51 (dd, 1H, $J_{2\text{-}1}$ = 6.2 Hz, $J_{2\text{-}3}$ = 7.5 Hz, H^{2}), 5.35 (ddd, 1H, $J_{28\text{-}29a} \cong J_{28\text{-}29b}$ = 6.0 Hz, $J_{28\text{-}27}$ = 11.0 Hz, CH=CH), 5.39 (ddd, 1H, $J_{27\text{-}26a} \cong J_{27\text{-}26b}$ = 6.0 Hz, $J_{27\text{-}28}$ = 11.0 Hz, CH=CH) ppm. ^{13}C NMR (CD_3OD) δ: 13.1, 22.3, 24.2, 24.6, 25.1, 25.5, 25.7, 26.2, 26.7, 26.7, 26.9, 28.8, 28.9, 28.9, 29.0, 29.1, 29.20, 29.22, 29.33, 29.34, 29.35, 29.4, 29.4, 31.7, 33.5, 53.0, 55.1, 63.1, 65.5, 70.8, 73.3, 73.6, 75.4, 76.4, 76.9, 77.6, 79.4, 103.1, 105.7, 108.4, 109.7, 109.9, 129.5, 129.5, 173.8 ppm. IR (Nujol): 2965, 1731, 1713 cm^{-1}.

2.2.2 General procedure for the synthesis of lactose fatty acid esters (*Z*)-6'-*O*-hexadec-9-enoyl- and (*Z*)-6'-*O*-tetracos-15-enoyl-4-*O*-(β-D-galactopyranosyl)-D-glucopyranose (4a,b).

Compounds **3a** or **3b** (0.43 mmol) were dissolved in tetrafluoroboric diethylether/water/acetonitrile (1:5:500) and the mixture was stirred at 30 °C for 2 h. The products precipitated during the reaction as white solid were subsequently filtered, washed with acetonitrile, and then dried. The purification by crystallization from methanol gave the desired compounds as white solids.

4a [(*Z*)-6'-*O*-Hexadec-9-enoyl lactose, lactose palmitoleate, URB1076]. Yield: 82% (0.305 g). Mp: modification of the physico-chemical state starting from 60 °C. ESI-MS: *m/z* 577 $(M-H)^-$, 596 $(M+NH_4)^+$. ^{1}H NMR (CD_3OD) δ: 0.91 (t, 3H, J = 7.0 Hz, CH_3), 1.25–1.45 [m, 16H, $(CH_2)_n$], 1.57–1.70 (m, 2H, CH_2CH_2COOR), 1.98–2.12 (m, 4H, $CH_2CH{=}CHCH_2$), 2.39 (t, 2H, J = 7.5 Hz, CH_2COOR), 3.42 (dd, 1H, $J_{2\text{-}1}$ = 3.5 Hz, $J_{2\text{-}3}$ = 9.5 Hz, H^2), 3.50 (dd, 1H, $J_{4\text{-}3} \cong J_{4\text{-}5}$ = 9.5 Hz, H^4), 3.51 (dd, 1H, $J_{9\text{-}10}$ = 3.0 Hz, $J_{9\text{-}8}$ = 9.8 Hz, H^9), 3.58 (dd, 1H, $J_{8\text{-}7}$ = 7.5 Hz, $J_{8\text{-}9}$ = 9.8 Hz, H^8), 3.75–3.96 (m, 4H, H^5, H^{11}, H^{6a}, H^{6b}), 3.79 (dd, 1H, $J_{3\text{-}4} \cong J_{3\text{-}2}$ = 9.5 Hz, H^3), 3.80 (dd, 1H, $J_{10\text{-}9}$ = 3.0 Hz, $J_{10\text{-}11}$ = 5.0 Hz, H^{10}), 4.26 (dd, 1H, $J_{12b\text{-}11}$ = 5.0 Hz, $J_{12b\text{-}12a}$ = 11.5 Hz, H^{12b}), 4.29 (dd, 1H, $J_{12a\text{-}11}$ = 6.5 Hz, $J_{12a\text{-}12b}$ = 11.5 Hz, H^{12a}), 4.35 (d, 1H, $J_{7\text{-}8}$ = 7.5 Hz, H^7), 5.09 (d, 1H, $J_{1\text{-}2}$ = 3.5 Hz, H^1), 5.32 (ddd, 1H, $J_{22\text{-}23a} \cong J_{22\text{-}23b}$ = 6.0 Hz, $J_{22\text{-}21}$ = 11.0 Hz, CH=CH), 5.37 (ddd, 1H, $J_{21\text{-}20a} \cong J_{21\text{-}20b}$ = 6.0 Hz, $J_{21\text{-}22}$ = 11.0 Hz, CH=CH) ppm. ^{13}C NMR (CD_3OD) δ: 13.0, 22.3, 24.5, 26.7, 28.6, 28.8, 28.9, 29.4, 31.5, 33.4, 60.7, 63.2, 68.8, 69.8, 70.8, 71.8, 72.2, 72.9, 73.2, 80.8, 92.3, 103.9, 129.4, 129.5, 174.0 ppm. IR (Nujol): 3404, 2951, 1735, 1711 cm^{-1}.

4b [(*Z*)-6'-*O*-tetracos-15-enoyl lactose, lactose nervonate, URB1077]. Yield: 93% (0.276 g). Mp: modification of the physico-chemical state starting from 60 °C. ESI-MS: *m/z* 690 $(M-H)^-$, 709 $(M+NH_4)^+$. ^{1}H NMR (DMSO) δ: 0.86 (t, 3H, J = 6.5 Hz, CH_3), 1.15–1.35 [m, 32H, $(CH_2)_n$], 1.47–1.58 (m, 2H, CH_2CH_2COOR), 1.94–2.04 (m, 4H, $CH_2CH{=}CHCH_2$), 2.30 (t, 2H, J = 7.5 Hz, CH_2COOR), 3.17 (ddd, 1H, $J_{2\text{-}1}$ = 4.0 Hz, $J_{2\text{-}OH2}$ = 7.0 Hz, $J_{2\text{-}3}$ = 9.5 Hz, H^2), 3.27 (dd, 1H, $J_{4\text{-}3} \cong J_{4\text{-}5}$ = 9.5 Hz, H^4), 3.33–3.37 (m, 2H, H^8, H^9), 3.57 (dd, 1H, $J_{3\text{-}2} \cong J_{3\text{-}4}$ = 9.5 Hz, H^3), 3.60–3.67 (m, 3H, H^{6a}, H^{6b}, H^{10}), 3.68–3.77 (m, 2H, H^5, H^{11}), 4.08 (dd, 1H, $J_{12b\text{-}11}$ = 4.5 Hz, $J_{12b\text{-}12a}$ = 11.5 Hz, H^{12b}), 4.16 (dd, 1H, $J_{12a\text{-}11}$ = 8.5 Hz, $J_{12a\text{-}12b}$ = 11.5 Hz, H^{12a}), 4.20–4.27 (m, 2H, H^7, OH^3), 4.41 (dd, 1H, $J_{OH6\text{-}6a} \cong J_{OH6\text{-}6b}$ = 6.0 Hz, OH^6), 4.54 (d, 1H, $J_{OH2\text{-}2}$ = 7.0 Hz, OH^2), 4.78 (d, 1H, $J_{OH10\text{-}10}$ = 5.0 Hz, OH^{10}), 4.85 (br s, 1H, OH), 4.90 (dd, 1H, $J_{1\text{-}OH1} \cong J_{1\text{-}2}$ = 4.0 Hz, H^1), 5.15 (br s, 1H, OH), 5.31 (ddd, 1H, $J_{22\text{-}23a} \cong J_{22\text{-}23b}$ = 6.0 Hz, $J_{22\text{-}21}$ = 11.0 Hz, CH=CH), 5.34 (ddd, 1H, $J_{21\text{-}20a} \cong J_{21\text{-}20b}$ = 6.0 Hz, $J_{21\text{-}22}$ = 11.0 Hz,

CH=CH), 6.33 (d, 1H, J_{OH1-1} = 4.0 Hz, OH^1) ppm. ^{13}C NMR (DMSO) δ: 14.4, 22.6, 24.8, 27.01, 27.02, 29.0, 29.02, 29.1, 29.2, 29.22, 29.29, 29.31, 29.4, 29.50, 29.52, 29.53, 29.6, 31.8, 33.8, 61.0, 63.7, 68.7, 70.2, 70.8, 71.7, 72.7, 72.9, 73.3, 81.6, 92.5, 104.0, 130.1, 130.1, 173.4 ppm. IR (Nujol): 3415, 2960, 1731, 1712 cm^{-1}.

2.3 Cell culture

Caco-2 cells were cultured to confluence in 75 cm^3 flasks at 5% CO_2 and 37 °C. Once confluent, they were detached from the flasks and seeded on filter inserts (Transwell®) at 100,000 cells/cm^2. Cells were maintained at 5% CO_2, 37 °C in DMEM supplemented with FBS (10%) antibiotic/antimycotic and L-glutamine, which was changed regularly (every other day). Cell growth and tight junction formation was assessed by transepitelial electrical resistance (TEER) measurements. Cell layers were used for TEER and permeability experiments following 21 days culture on Transwell inserts.

2.4 MTS toxicity assay

The MTS colorimetric assay was performed to evaluate the effect of surfactants on cell viability. Caco-2 cells were seeded on 96-well plates at 10,000 cells per well and cultured in DMEM for 24 h. Prior to the assay, cell medium was removed and replaced with surfactant samples at the following concentrations: 0.00625 mg/mL, 0.0125 mg/mL, 0.025 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL and 0.8 mg/mL in HBSS. Triton X-100 (0.1%, v/v in HBSS) and HBSS were used as a positive and negative control, respectively. Cells were incubated (at 37 °C, 5% CO_2) with samples and controls for a period of 3 h. Samples (and controls) were then removed and cells washed with phosphate-buffered saline (PBS). The MTS assay was subsequently conducted according to the manufacturer's instructions, with four repeats for each sample.

The relative cell viability (%) was calculated using the following equation:

$$Relative\ Viability = \frac{S - T}{H - T} \times 100 \quad \textbf{Eq. (1)}$$

Where: S is the absorbance of the tested samples, T is the absorbance of cells incubated with Triton X-100, and H is the absorbance of cells incubated with HBSS.

2.5 TEER experiments

Caco-2 cell monolayers with a TEER ≥800 Ωcm^2 were used in these experiments. Prior to the sample application, cell medium was removed and replaced with HBSS. Cells were equilibrated in HBSS (incubated at 37 ºC, 5% CO_2) for 30 min, following which TEER was measured; this was treated as the baseline TEER. Surfactants solutions at 0.0125-0.1 mg/mL concentration range were then applied to the apical side of the cell monolayers and cells were incubated with the samples for 3 h. Chitosan at 0.1 mg/mL was employed for comparison as an example of a compound with well-documented ability to open epithelial tight junctions, and as a result, decrease TEER. TEER was measured every 30 min for 3 h in the presence of the tested surfactant samples. The samples were removed after 3 h and cells washed extensively with PBS. Cell medium was then added to both sides of the cell monolayers and cells incubated with the culture medium (DMEM) overnight. A further measurement of TEER was taken (with cells bathed in medium) 24 h following the exposure of the cells with surfactants to establish whether the changes in TEER (if any) were reversible. TEER was measured using an EVOM Voltohmmeter (World Precision Instruments, UK), equipped with a pair of chopstick electrodes. Background TEER due to the filter (~100 to 110 Ωcm^2) was deducted from the measurements in all cases. All experiments were performed in triplicates.

2.6 Permeability experiments

FITC-OVA was used as model of a protein drug. Caco-2 cells were cultured on filters as described above and only cell monolayers with TEER ≥800 Ωcm^2 were used for the purpose of this experiment. Prior to the sample application, culture medium was removed and the cell layers washed with PBS. Cells were then equilibrated in HBSS for 30-45 min. Surfactant solutions, at the final concentrations of 0.2, 0.1 and 0.05 mg/mL and FITC-OVA of 100 μg/mL, respectively, in HBSS, were then applied together to the apical side of the cells. Basolateral solution was sampled (100 μL volumes) at 30, 60, 90, 120, 150 and 180 min after sample application and the

sampled volume replaced with fresh HBSS. Sampled FITC-OVA was quantified by fluorescence, using a Tecan M200 Pro plate reader. After the final sampling, the cell layers were then washed with PBS and TEER measured in order to ensure that the cell layer integrity was not compromised during the permeability experiments and that cells recover. The permeability of FITC-OVA is expressed as the apparent permeability coefficient (P_{app}), calculated using the following equation:

$$P_{app} = \left(\frac{\Delta Q}{\Delta t}\right) \times \left(\frac{1}{A \times C_0}\right) \quad \textbf{Eq. (2)}$$

P_{app}, apparent permeability (cm/s); $\Delta Q/\Delta t$, permeability rate (amount of FITC-OVA traversing the cell layers over time); A, diffusion area of the layer (cm_2); C_0, apically added FITC-OVA concentration. The experiment was conducted in triplicates.

2.7 Bacterial strains and culture conditions

Eight reference human pathogens were used in this study, *Escherichia coli* O157:H7 ATCC 35150, *Listeria monocytogenes* ATCC 7644, *Salmonella enteritidis* ATCC 13076, *Enterococcus fecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 43387, *Yersinia enterocolitica* ATCC 27729, and *Candida albicans* ATCC 14053. All the strains were routinely maintained in Tryptic Soy Agar (TSA, Oxoid, Milan, Italy) at 37 °C, while stock cultures were keep at -80 °C in Nutrient broth (Oxoid) with 15% of glycerol.

2.8 Determination of MICs

MICs determination of lactose palmitoleate and lactose nervonate was performed by microdilution method. For the tests, each compound (1.28 mg) was dissolved in DMSO (1 mL). Several colonies of each bacterial strain were picked and inoculated in sterilized Mueller-Hinton broth (MHB) (Oxoid) (10 mL) and incubated at 37 °C for 18–24 h. Bacterial suspensions were adjusted by spectrophotometer to a turbidity corresponding to 10^6 cfu/mL (OD_{610nm} 0.13-0.15) and each bacterial suspension (100 µL) was added in wells of the 96-well plate together with the appropriate volumes of the test solution to obtain final concentrations of 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5 µg/mL. Two rows of the 96-well plate were used for positive (bacteria alone) and negative controls (MHB alone), respectively. Gentamicin (128-0.125 µg/mL) and a

standard preservative mixture (methylparaben and propylparaben, ratio 9:1) (1024-0.5 μg/mL) were added as internal controls. Preliminary assays with DMSO were performed to exclude its possible bacteriostatic and/or bactericidal activity; therefore, volumes of DMSO solutions added in each well never exceeded 5% (v/v) of the final total volume. MICs were defined as the lowest concentration of compound inhibiting the visible bacterial growth after 24 h of incubation. All the experiments were performed in duplicate.

2.11 Time kill experiments against food-borne pathogens

To evaluate the antimicrobial activity of lactose palmitoleate and lactose nervonate, time-kill experiments against food-borne pathogens, here represented by *E. coli* O157: H7 ATCC 35150, *L. monocytogenes* ATCC 7644, and *S. enteritidis* ATCC 13076, were performed. For this, pathogens strains were grown overnight in 20 mL of MHB at 37 °C and, after incubation, 500 μL (about 10^7 cfu/mL, as previously determined by spectrophotometer) of each pathogen suspension were incubated in 24-well culture plates with 500 μL of MBH with lactose palmitoleate or lactose nervonate at MIC and 2MIC concentrations. Several wells were inoculated with 500 μL of each pathogen suspension in 500 μL of MBH as controls.

At baseline, and after 3, 6 and 24 h of incubation, one aliquot of each sample was aseptically removed, serially diluted in physiological saline solution, and plated on TSA plates for colonies forming units enumeration (cfu/mL). Volumes of DMSO solution added in each well never exceeded 1% (v/v) of the final volume. All the experiments were performed in duplicate.

2.12 Statistical analysis

Statistical analysis was performed using Prism version 5.0 (GraphPad Inc., USA). The assumptions for parametric test were cheeked prior to carry out the analysis. To compare the numbers of bacteria recovered in time-kill experiments after exposure to lactose palmitoleate and lactose nervonate, one-way analysis of variance (ANOVA) with Bonferroni post-test was performed; when the assumptions for parametric test were not respected, Kruskall-Wallis non parametric test with Dunn's multiple comparison test was applied. P values < 0.05 were considered to be statistically significant.

3. Results

3.1 Cell viability

Figure 1 shows the effect of tested compounds, applied within a broad dose range, on Caco-2 cell viability. Lactose palmitoleate did not display notable toxicity to Caco-2 cells regardless of the applied concentration (cell viability ranged from 88% to 141%) and no dose-dependent effect was apparent. Similarly, lactose nervonate did not show a concentration-dependent effect, but with this surfactant the majority of the tested concentrations were associated with a decrease in cell viability, which was most apparent with 0.05 mg/mL.

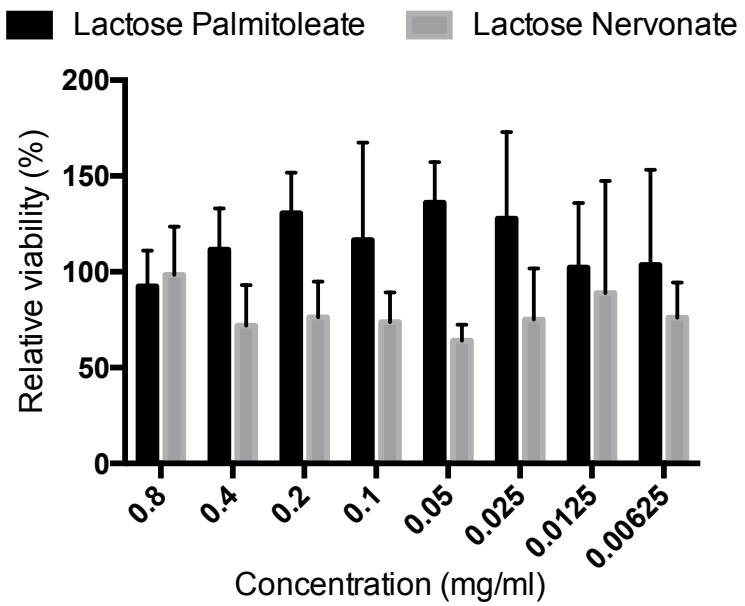


Figure 1. Effect of lactose palmitoleate and lactose nervonate surfactants on relative Caco-2 cell viability, as determined by the MTS metabolic activity assay. Surfactants were applied at 0.00625 mg/mL, 0.0125 mg/mL, 0.025 mg/mL, 0.05 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL and 0.8 mg/mL. Relative viability calculated by normalising against negative control, Hank's Balanced Salt Solution (HBSS) and positive control, 0.1% v/v Triton X-100 in HBSS. Data shown as the mean ± SD (n=6).

3.2 TEER

Figure 2 shows the effect of lactose palmitoleate and lactose nervonate surfactants on Caco-2 monolayer TEER. The concentration range tested for their impact on epithelial TEER was 0.0125-0.1 mg/mL, which is in fact well below 0.8 mg/mL, the

dose found to be not toxic to Caco-2 cells (Figure 1). Chitosan was incorporated in this experiment as a TEER-lowering compound to provide a comparison.

The data shows that both lactose palmitoleate and lactose nervonate decreased Caco-2 monolayer TEER at all tested doses. For lactose palmitoleate, there is a sharp decrease in TEER, with maximal decrease by 62-68% of the baseline value (depending on the concentration), observed 2.5 h post application (Figure 2A). Compared to chitosan, although a lower minimal TEER compared to the surfactants is apparent with chitosan, the maximal decrease amounted to 62% of the baseline value. With lactose nervonate, a more gradual decrease in TEER was observed compared to both lactose palmitoleate and chitosan. TEER reached a minimal value 3 h post application, which equated to a drop by 41-65%, depending on the concentration (highest dose exerting the largest TEER decrease). With both surfactant compounds, TEER reversed to original (pre-application) values, confirming no long-lasting effect on cell toxicity, tight junctions and cell monolayer integrity, while the TEER of chitosan-treated cells showed partial TEER reversibility (to 64% of the baseline value).

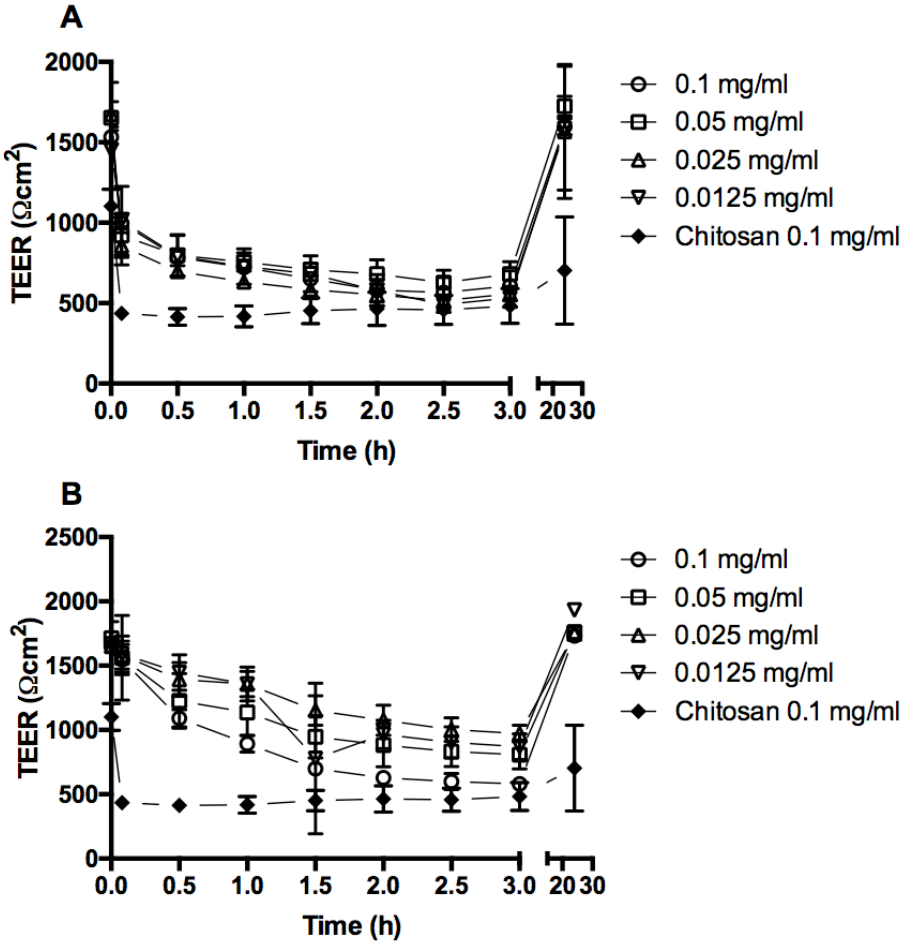


Figure 2. Effect of lactose esters on Caco-2 cell monolayer transepithelial electrical resistance (TEER). A) Lactose palmitoleate and B) Lactose nervonate. Surfactants were applied to confluent cell monolayers at a concentration of 0.1, 0.05, 0.025 and 0.0125 mg/mL. Data are expressed as % of the baseline TEER and presented as the mean ± SD (n=3).

3.3 Permeability studies

The effect of surfactants lactose palmitoleate and lactose nervonate on the permeability (apparent permeability coefficient) of a model protein, FITC-OVA (Mw ~45,000 Da), is shown in Figure 3 (A and B, respectively). The compounds were applied to Caco-2 monolayers at 0.2 mg/mL, 0.1 mg/mL and 0.05 mg/mL. Applied at 0.2 mg/mL, lactose palmitoleate enhanced FITC-OVA permeability 11.5-fold (Figure 3A). The next lower dose (0.1 mg/mL) increased FITC-OVA permeability, but this increase did not reach statistical significance. The lowest applied concentration of lactose palmitoleate did not influence FITC-OVA permeability.

With lactose nervonate (Figure 3B), the highest and lowest used doses (0.2 and 0.05 mg/mL, respectively) did not induce a statistically significant effect on FITC-OVA permeability. The 0.1 mg/mL dose, however, led to a 2.5-fold enhancement of FITC-OVA permeability. **It is not presently clear why at 0.1 mg/ml lactose nervonate induced a higher permeability than the higher dose of 0.2 mg/ml. However, this may be related to the complex relationship between surfactant concentration and their behaviour in solution (including CMC) and, in turn, interaction with the biological systems.**

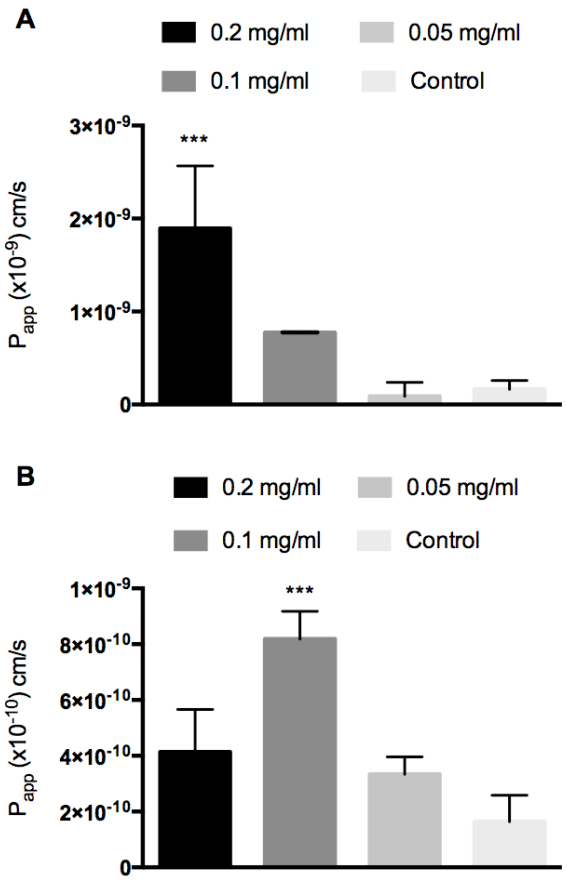


Figure 3. Effect of lactose esters on ovalbumin permeability across Caco-2 cell monolayers. A) Lactose palmitoleate and B) Lactose nervonate. Surfactants were applied to confluent cell monolayers at 0.2 mg/ml, 0.1 mg/mL and 0.05 mg/mL. Data are expressed as apparent permeability coefficient (P_{app}) and presented as the mean ± SD (n=3).

3.4 Antimicrobial activities of lactose palmitoleate and lactose nervonate

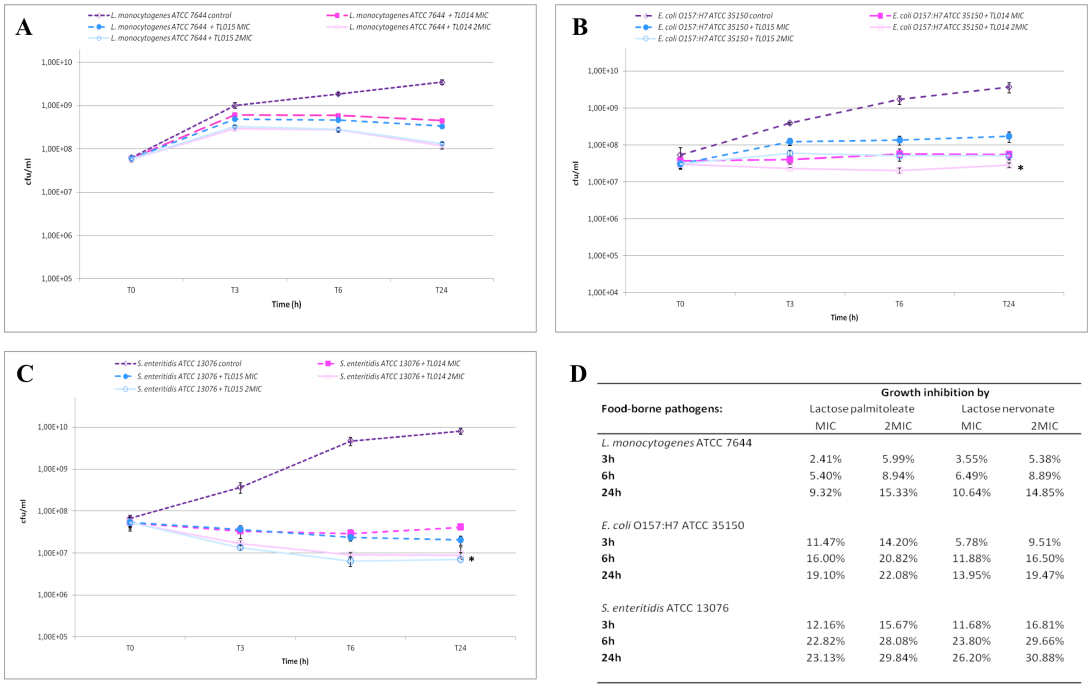
The antimicrobial activities of lactose palmitoleate and lactose nervonate were evaluated by determining the MIC, and subsequently carrying out time-kill experiments against food-borne pathogens. MICs of lactose palmitoleate and lactose nervonate against *Escherichia coli* O157:H7 ATCC 35150, *Listeria monocytogenes* ATCC 7644, *Salmonella enteritidis* ATCC 13076, *Enterococcus fecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 43387, *Yersinia enterocolitica* ATCC 27729 and *Candida albicans* ATCC 14053 were tested according to the National Committee for Clinical Laboratory Standards (NCCLS) document M100-S12 method. The relative data are shown in **Table 1**.

Lactose nervonate showed the greatest antimicrobial activity against the three food-borne pathogens included in this study, *Escherichia coli* O157:H7 ATCC 35150,

412 *Listeria monocytogenes* ATCC 7644, and *Salmonella enteritidis* ATCC 13076, with
413 MIC values of 64 µg/mL.
414 Lactose palmitoleate showed similar MIC values of 64 µg/mL toward *Escherichia*
415 *coli* O157:H7 ATCC 35150 and *Listeria monocytogenes* ATCC 7644, and a higher
416 MIC value (128 µg/mL) towards *Salmonella enteritidis* ATCC 13076. The MICs
417 values of lactose palmitoleate and lactose nervonate against the others tested
418 microorganisms were similar to those reported against the food-borne pathogens.
419 With regards to internal controls, gentamicin inhibited microbial growth with the
420 lowest MIC value of 4 µg/mL for *Salmonella enteritidis* ATCC 13076 and the highest
421 MIC value of 128 µg/mL for *Escherichia coli* O157:H7 ATCC 35150, while parabens
422 mixture showed MIC values >1024 µg/mL for all the examined bacterial species.
423
424
425 **Table 1**. MIC values (µg/mL) of the tested compounds against selected bacterial strains.

Target microrganisms	MICs (µg/mL)			
	Lactose palmitoleate	Lactose nervonate	Gentamicin	Parabens
E. coli O157:H7 ATCC 35150	64	64	128	>1024
L. monocytogenes ATCC 7644	64	64	8	>1024
S. enteritidis ATCC 13076	128	64	4	>1024
E. faecalis ATCC 29212	64	64	64	>1024
P. aeruginosa ATCC 9027	128	128	16	>1024
S. aureus ATCC 43387	128	128	16	>1024
Y. enterocolitica ATCC 27729	64	64	8	>1024
C. albicans ATCC 10231	64	64	NA	>1024

426 NA: not applicable
427
428 Results of time–kill experiments with lactose palmitoleate and lactose nervonate at
429 their respective MIC and 2MIC concentrations against *E. coli* O157:H7 ATCC 35150,
430 *L. monocytogenes* ATCC 7644, and *S. enteritidis* ATCC 13076 are summarized in
431 **Fig. 4**.



D

Food-borne pathogens:	Growth inhibition by Lactose palmitoleate MIC	Lactose palmitoleate 2MIC	Lactose nervonate MIC	Lactose nervonate 2MIC
L. monocytogenes ATCC 7644				
3h	2.41%	5.99%	3.55%	5.38%
6h	5.40%	8.94%	6.49%	8.89%
24h	9.32%	15.33%	10.64%	14.85%
E. coli O157:H7 ATCC 35150				
3h	11.47%	14.20%	5.78%	9.51%
6h	16.00%	20.82%	11.88%	16.50%
24h	19.10%	22.08%	13.95%	19.47%
S. enteritidis ATCC 13076				
3h	12.16%	15.67%	11.68%	16.81%
6h	22.82%	28.08%	23.80%	29.66%
24h	23.13%	29.84%	26.20%	30.88%

Figure 4. Antimicrobial activity of lactose palmitoleate and lactose nervonate at MIC and 2MIC concentration s in time-kill experiments against food-borne pathogens *L. monocytogenes* ATCC 7644 (A), *E. coli* O157:H7 ATCC 35150 (B), *S. enteritidis* ATCC 13076 (C) and relative growth inhibition percentages (D). Data represent mean values of three independent experiments performed in duplicate and asterisks values statistically significant ($P < 0.05$, Kruskall-Wallis non-parametric test with Dunnett's multiple comparison test).

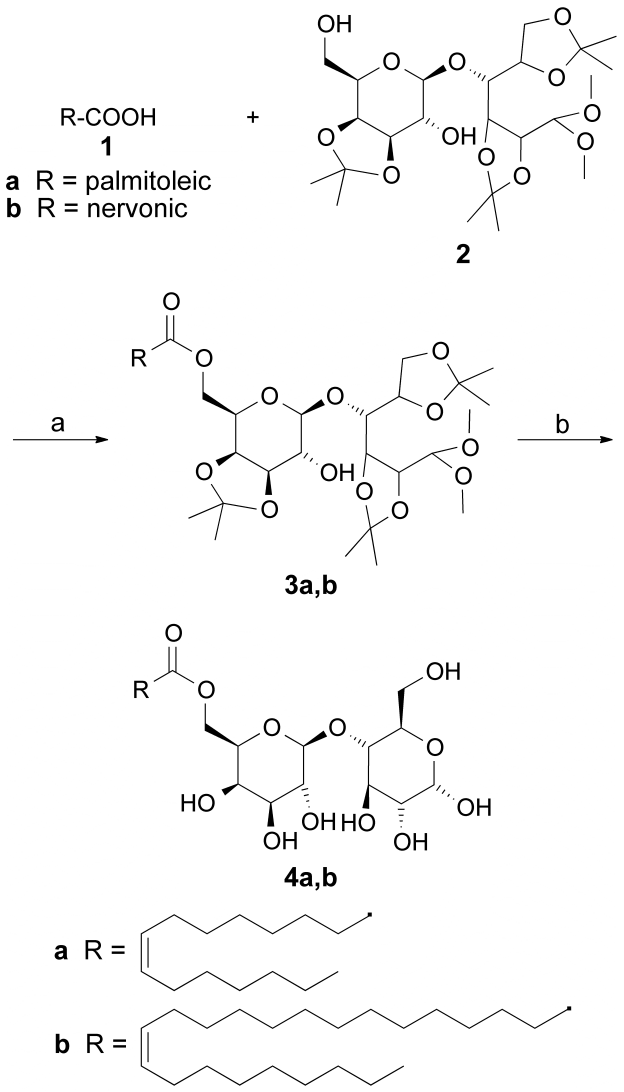
In general, the antimicrobial effect of these compounds was confirmed on the tested food-borne pathogens with a cfu/mL reduction in all the samples containing lactose palmitoleate or lactose nervonate at different concentrations (MIC and 2MIC) in comparison to the relative control samples (**Fig. 4a-c**). In particular, the viability of *E. coli* O157:H7 ATCC 35150 decreased significantly to 7.70 log cfu/mL after 24 h of incubation with lactose palmitoleate at 2MIC, compared to 9.56 log cfu/mL of the control one (**Fig. 4b)**. Similarly, the viability of *S. enteritidis* ATCC 13076 was significantly reduced after 24 h of incubation with lactose palmitoleate and lactose nervonate at 2MIC with 6.95 and 6.85 log cfu/mL, respectively, compared to 9.90 log cfu/mL of the relative control (**Fig. 4c)**.

Both the tested substances induced a bacterial growth reduction during the entire incubation time, with an increased rate from 6 to 24 h. The highest values of growth inhibition, 30.88 and 29.84%, were obtained for *S. enteritidis* ATCC 13076 after 24 h of incubation with lactose palmitoleate and lactose nervonate at 2MIC value concentration, respectively (**Fig. 4d**). Similar percentages of growth inhibition were

also observed for *S. enteritidis* ATCC 13076 after 6 h incubation with lactose palmitoleate and lactose nervonate at MIC concentration (29.66 and 28.08%, respectively). With regards to *E. coli* O157:H7 ATCC 35150, growth inhibitions amounting to 22.08 and 19.47% were evidenced after 24 h incubation with lactose palmitoleate and lactose nervonate at 2MIC value concentration, respectively. Lower percentages of growth inhibition were obtained with *L. monocytogenes* ATCC 7644, with 15.33 and 14.85% of growth inhibition after 24 h incubation in the presence of lactose palmitoleate and lactose nervonate at 2MIC concentration, respectively **(Fig. 4 d)**.

4. Discussion

Different chemical or enzymatic synthetic strategies have been adopted to produce biodegradable, biocompatible and eco-friendly sugar-based materials with interesting properties, including ability to act as permeability enhancers and/or antimicrobial agents [2,17–21]. Among them, the enzymatic production of sucrose esters represent a route to obtain a promising class of compounds with multiple applications, already marketed in different fields [22,23]. Lactose palmitoleate (URB1076) and lactose nervonate (URB1077) were synthesized from palmitoleic acid (**1a**) or nervonic acid (**1b**) following a literature procedure based on a specific lipase as a catalyst, namely Lipozyme® [19], and requiring a preventive step for the protection of disaccharide derivative lactose to obtain LTA (**2**) [19] (Scheme 1). The final step proceeded through the deprotection of the acetalic adducts **3a,b** to obtain the desired compounds **4a,b** (Scheme 1).



Scheme 1. Reagents and conditons: (a) toluene, 75 °C, 12 h; (b) $HBF_4{\cdot}Et_2O$, CH_3CN, 30 °C, 4 h.

The use of the surfactant described is of potential high value due to their biological effectiveness at low concentrations and metabolism in vivo. This situation leads to non-toxic metabolites, particularly when the molecules obtained by ester bond hydrolysis are sugar and fatty acid derivatives such as those studied here.

Regarding cell toxicity, it is interesting to consider that both lactose palmitoleate and lactose nervonate did not show marked toxicity to Caco-2 cells, even with a relatively high application dose (0.8 mg/mL). Furthermore, no dose-dependency was apparent. The absence of significant cell toxicity with surfactant compounds, especially at doses used here, is rare. For example, Vllasaliu et al. previously evaluated alkylmaltosides (three units sugar and linear fatty chains from C12 to C14) for their absorption enhancing property. Using a combination of methods, they demonstrated that these

surfactants produced a significant level of toxicity in bronchial epithelial cells, Calu-3, with concentration of surfactant that caused 50% cell death (IC_{50}) values between 0.0031-0.0065% w/v for the three representative compounds tested. In another example, Warisnoicharoen et al. studied the toxicity of nonionic surfactants polyoxyethylene-10-oleyl ether (C18:1E10), polyoxyethylene-10-dodecyl ether, and *N*,*N*-dimethyldodecylamine-*N*-oxide in bronchial cells and obtained IC_{50} values ranging between 0.06-0.08 mg/mL [24].

Concerning the permeability enhancement activity, a wide range of ionic and non-ionic surfactants have been explored for their potential use as mucosal absorption enhancers. However, experience suggests that the use of surfactants as permeability enhancers is associated with cell toxicity [25–28], as discussed above, which severely limits their application. Of note is the emergence of alkylmaltosides, which have been clinically proposed for nasal delivery (e.g. Intraveil®). They are being explored commercially due to evidence of increased systemic bioavailability of peptides and proteins when included in nasal or ocular formulations [7,29] or when evaluated on Caco-2 and rat intestinal mucosal tissue [30].

Studies exploring the use of surfactants as mucosal absorption enhancers predominantly employ relatively low molecular peptides and proteins. However, we were interested to determine whether the permeability of OVA, as an exemplar protein of ~45 kDa, is improved in an intestinal model with the compounds synthesized here. A permeability enhancement ratio of 11.5 achieved with lactose palmitoleate is remarkable considering the molecular size of OVA. Perhaps even more remarkable is the fact that a clear permeability increasing effect is not mirrored by a notable change in TEER. The combination of findings therefore points to a transcellular rather than paracellular effect with lactose palmitoleate. These findings are in agreement with a recent study by Kiss et al. [22], which reported that non-toxic concentrations of sucrose esters significantly enhanced the permeability of atenolol and fluorescein across Caco-2 monolayers. In that study, however, the surfactants caused a reduction in TEER, but, interestingly, the morphology of tight junctions remained unaffected. The authors of this study concluded that sucrose ester surfactants act as absorption enhancers through an effect on both the transcellular and paracellular routes, with a clearly demonstrated effect on elevation of plasma membrane fluidity, which was suggested as a cause of increased transcellular passage of molecules. Overall, the permeability data is important within the context of non-

invasive delivery of peptide and protein therapeutics, as well as vaccine delivery (OVA is in fact a routinely used model vaccine antigen).

From the pharmaceutical to the cosmetic and food fields, the need of developing safe and efficient preservatives has been growing very rapidly, particularly to find alternatives to parabens. Different sugars derivatives have been proposed to achieve this goal, starting from monosaccharides to polysaccharides as glycosidic moieties. Among them, alkylated oligomaltosides (i.e. maltoside and maltotrioside) demonstrated a valuable alternative with good antimicrobial activity explained by the inhibition of the microbial enzymatic metabolism. Due to the low solubility of these compounds the authors conducted the experiments in DMSO and the results highlighted a higher microbial inhibition for di- and polysaccharide than monosaccharide derivatives [31].

In our study, the antibacterial activities of two sugar fatty acid esters, lactose palmitoleate and lactose nervonate, against several different human pathogens were evaluated. MICs of lactose palmitoleate and lactose nervonate, ranging from 64 to 128 μg/mL, evidenced a greater antibacterial property compared to the parabens mixture, with MIC values >1024 μg/mL. According to other authors who have tested the antibacterial efficacy of alkylated oligomaltosides [31], our findings highlight the potential use of lactose palmitoleate and lactose nervonate sugar esters as alternative preservatives to the commonly employed ones, such as parabens.

Moreover, in time-kill experiments performed toward selected food-borne pathogens, higher concentrations (2MIC values) of lactose palmitoleate and lactose nervonate were able to inhibit the growth of these bacteria, with a variable degree of antibacterial activity. For both the tested compounds, a bacteriostatic effect toward *L. monocytogenes* ATCC 7644 at each time point was observed, while after 24 h of incubation with lactose palmitoleate and lactose nervonate the numbers of viable *E. coli* O157:H7 ATCC 35150 and *S. enteritidis* ATCC 13076 were noticeably lower than the initial values. These data are in agreement with those of other researchers [3], which referred a strong antibacterial activity of sugar esters against food-borne pathogens. The results obtained here are interesting and encourage further studies in order to fully understand the antibacterial efficacy of lactose palmitoleate and lactose nervonate against other food-borne pathogens and their interactions with food ingredients, hence verifying their real application to control bacterial growth in food systems.

5. Conclusions

The study presented here reports novel sucrose ester-based surfactant compounds with a good toxicity profile, as determined by the MTS assay and evaluation of the effect on the epithelial barrier integrity (TEER investigations). The compounds were tested for and clearly shown to display a combination of macromolecular absorption enhancing and antimicrobial properties. This is important considering the toxicity profile of the compounds demonstrated here, as these properties are often associated with unacceptable toxicity. This work therefore clearly indicates that detailed evaluation of these compounds with potential use as absorption enhancers and/or alternative preservatives is warranted in the future.

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